Structure of the quinoprotein glucose dehydrogenase of *Escherichia coli* modelled on that of methanol dehydrogenase from *Methylobacterium extorquens*

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The structure of methanol dehydrogenase (MDH) at 0.194 nm (1.94 Å) has been used to provide a model structure for part of a membrane quinoprotein glucose dehydrogenase (GDH). The basic superbarrel structure is retained, along with the tryptophan-docking motifs. The active-site regions are similar, but there are important differences, the most important being that GDH lacks the novel disulphide ring structure formed from adjacent cysteines in MDH; in GDH the equivalent region is occupied by His-262. Because of the overall similarities in the active-site region, the mechanism of action of GDH is likely to be similar to that of MDH. The differences in co-ordination to the cation and bonding to the pyrrolo-quinoline quinone (PQQ) in the active site may explain the relative ease of dissociation of the prosthetic group from the holo-GDH. There are considerable differences in the external loops, particularly those involved in formation of the shallow funnel leading to the active site, the configuration of which influences substrate specificity. The proposed model is consistent in many respects with previous proposals for the active-site structure based on the effects of chemical modification on binding of PQQ and enzymic activity.

INTRODUCTION

Quinoprotein dehydrogenases containing pyrrolo-quinoline quinone (PQQ) as their prosthetic group include methanol dehydrogenase (MDH) from methylotrophic bacteria [1–3], the quinohaemoprotein alcohol dehydrogenase (ADH) from acetic acid bacteria [4] and the glucose dehydrogenase (GDH) from *Gluconobacter* [5,6], *Acinetobacter calcoaceticus* [7–9], *Pseudomonas fluorescens* [10,11] and *Escherichia coli* [12,13]. Although all these quinoproteins may be assayed using artificial electron acceptors such as phenazine ethosulphate, they differ with respect to their physiological electron acceptors. MDH is a soluble periplasmic enzyme with cytochrome c_L as the electron acceptor [14], whereas ADH is a multisubunit enzyme with a quinohaemoprotein subunit in which electrons pass from $PQQH_2$ to a haem C on the same subunit [4]. In contrast with these two enzymes, haem C is not involved in the activity of GDH which is a membrane-bound monomer involved in oxidation of glucose to gluconic acid in the periplasm; electrons pass directly from $PQQH_2$ to ubiquinone in the membrane [6,11,15]. In addition to this GDH, there is a second, soluble, quinoprotein GDH in *A. calcoaceticus* [9,16]; this has no sequence similarity to other quinoproteins [17] and will not be discussed further.

MDH is the only PQQ-containing quinoprotein for which an X-ray structure is available, and it is our recent high-resolution structure of the enzyme from *Methylobacterium extorquens* [3,18] that is used for the basis of the present paper. MDH has an $\alpha_2\beta_2$ tetrameric structure, each α-subunit (66 kDa) having a single molecule of PQQ and a Ca^{2+} ion. The small β-subunits (8.5 kDa) fold around the surface of the α-subunits. The α-subunit is a superbarrel made up of eight topologically identical four-stranded antiparallel β-sheets (W-shaped) arranged with radial symmetry like the blades of a propeller. The PQQ is buried in the interior of the superbarrel within a chamber that communicates with the exterior through a hydrophobic funnel-shaped depression in the surface. The floor of the active-site chamber is formed by the plane of a tryptophan residue, the ceiling being formed by a novel ring structure arising from a disulphide bridge between adjacent cysteine residues. A second important feature seen in the active site is a Ca^{2+} ion which plays a role in maintaining PQQ in the correct configuration and which may also be involved in the catalytic mechanism.

GDH is an intrinsic membrane protein which must be solubilized from the membrane before purification, and evidence from ultracentrifugation indicates that the enzyme is monomeric (about 87 kDa) [10,12]. The N-terminal region of GDH (residues 1–154) forms a membrane anchor with five transmembrane segments [19]; this region is likely to contain the ubiquinone-binding site. The remaining periplasmic region (residues 155–796) shows 26% identity of sequence with that of the α-subunit of MDH, indicating that GDH might have an essentially similar structure. GDH differs from MDH and ADH in many ways, however. Its substrate is the pyranose form of D-glucose (and other monosaccharides), the immediate product being the gluconolactone. Its electron acceptor is membrane ubiquinone [6,11,15], rather than haem C which is the electron acceptor for MDH and ADH whose substrates are primary alcohols. GDH differs from the other quinoproteins in ways that imply important differences with respect to its active site and its mechanism. In contrast with MDH, PQQ is relatively easily released from GDH, and the enzyme from *E. coli* and *Acinetobacter lwoffi* actually occurs as the apoenzyme [13,20]. Reconstitution of active enzyme is readily achieved by incubation with PQQ and Ca^{2+} [21,22], suggesting that this cation is involved in binding PQQ in the active site and may also be involved in the reaction mechanism as suggested for MDH [23]. That the mechanism

Abbreviations used: ADH, quinohaemoprotein alcohol dehydrogenase; GDH, glucose dehydrogenase; MDH, methanol dehydrogenase; PQQ, pyrrolo-quinoline quinone.

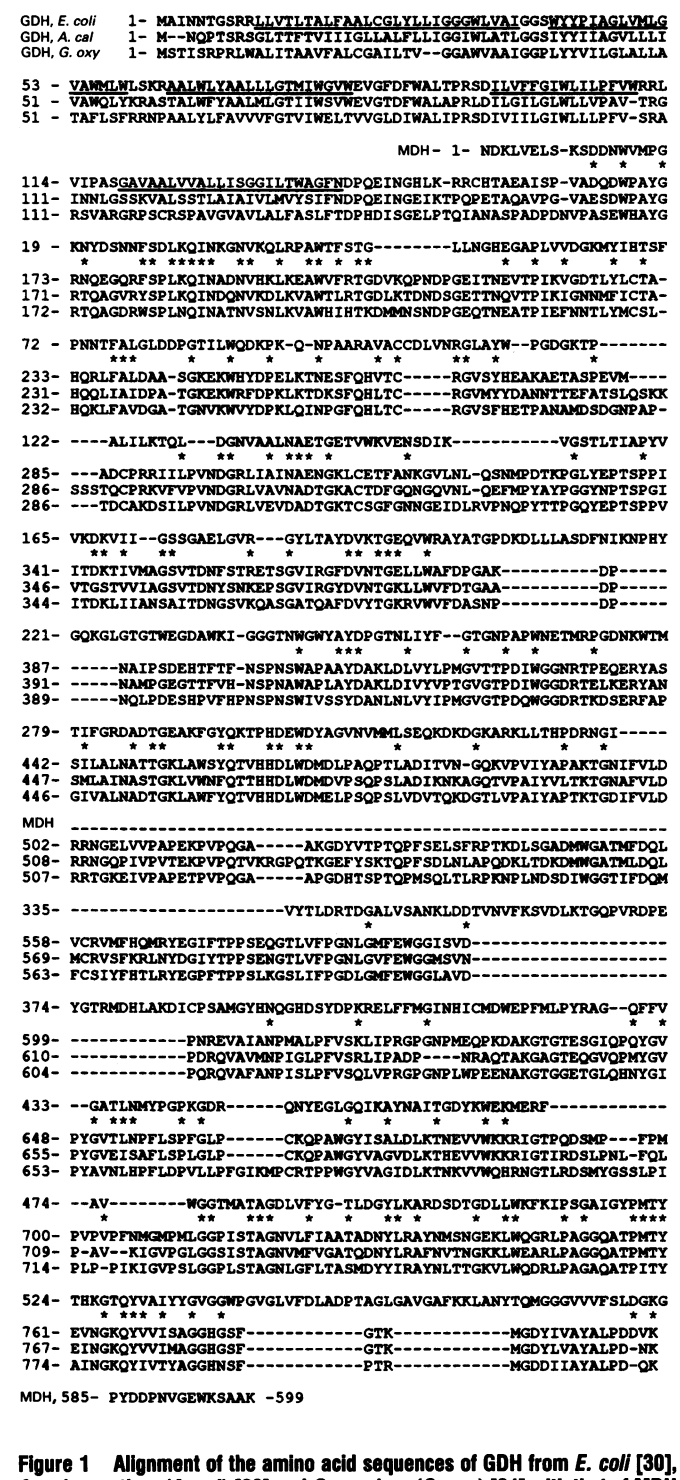

Figure 1 Alignment of the amino acid sequences of GDH from *E. coli* [30], *A. calcoaceticus* (*A. cal*) [29] and *G. oxydans* (*G. oxy*) [31] with that of MDH from *M. extorquens* [17,27,28]

The asterisks denote identical residues between the sequences of GDH from *E. coli* and MDH from *M. extorquens*. The underlined sequence indicates the proposed transmembrane regions in the N-terminal sequence of GDH. The sequence of MDH does not include the signal peptide which is cleaved from the preprotein during production of the final periplasmic protein [33].

might be different, however, is indicated by the fact that Mg^{2+} can replace Ca^{2+} (MDH cannot use Mg^{2+}) [21,24]. That there are further important differences in the mechanism is also suggested by chemical modification studies on GDH [25] and by sequence considerations, which indicate that the novel disulphide ring structure of MDH is absent from the active-site region of GDH [26].

This paper presents the results of modelling the sequence of the C-terminal region of the membrane GDH of *E. coli* onto the co-ordinates of the α-subunit of MDH, in order to provide a basis for interpretation of previous work on the active site of this enzyme, and for design of future work using the tools of site-directed mutagenesis.

MATERIALS AND METHODS

The sequences of the α- and β-subunits of MDH were those for the enzyme from *M. extorquens* [17,27,28]. The gene-derived protein sequences of three GDHs are available [29–32] (Figure 1); the primary sequence used for modelling GDH was that of the enzyme from *E. coli* [30]. Alignment and modelling was conducted using residues 1–595 of MDH and residues 155–796 of GDH. In the regions selected there was a 26% identity and 67% similarity of sequence (Figure 1). All analysis and modelling was performed using the program package Quanta/CHARMm. Sequence alignment was initially determined using a Needleman–Wunsch algorithm [34] and a protein sequence score matrix [35]. This alignment was then refined by hand where necessary assuming conservation of residues known to be structurally significant in the β-sheet superbarrel of MDH in GDH. Figure 1 shows this alignment along with the sequences of GDH from *A. calcoaceticus* [29] and *Gluconobacter oxydans* [31]. Wherever possible insertions or deletions were incorporated opposite the loop regions of MDH rather than in the β-sheet W motifs. The backbone co-ordinates of aligned residues in MDH were then mapped to the equivalent amino acids in GDH. Where insertions or deletions occurred in GDH a fragment database [36] was used to search for short sequences that overlapped with the structure either side of the unknown region and that contained the same number of residues. The best-fit fragment was then used to model the GDH. After definition of the backbone co-ordinates, the validity of the emerging structure was assessed using the Protein Health module in Quanta. The irregularities identified were either remodelled manually or by reusing the fragment database. Once the backbone residues had been defined, the side-chain co-ordinates of the identical amino acids were also copied from MDH to GDH. Quanta/CHARMm was then used to generate side chains for the non-identical amino acids in their most likely rotameric form. Major side-chain clashes were removed manually, then CHARMm was used to perform an energy minimization applying constraints to preserve the basic backbone structure as in MDH. As the energy minimization is based on absolute zero, and the MDH structure was determined at about 20 °C, the MDH structure was also minimized using similar constraints to those used to model the GDH. The final energies obtained were of the same order of magnitude, and the MDH did not show major structural perturbations during minimization. The final structure was checked for abnormalities and inconsistencies using the Protein Health option in Quanta.

RESULTS AND DISCUSSION

Figure 2 shows the overall structure of the model GDH compared with the structure of MDH and the model structure of the N-terminal region of ADH (from ref. [37]). The regions of greatest sequence similarity are the eight β-sheet regions (the W motifs or propeller blades) and these sequences in GDH model very well on to the MDH structure, and also the ADH structure. These β-sheet W motifs are held together by special tryptophan-docking

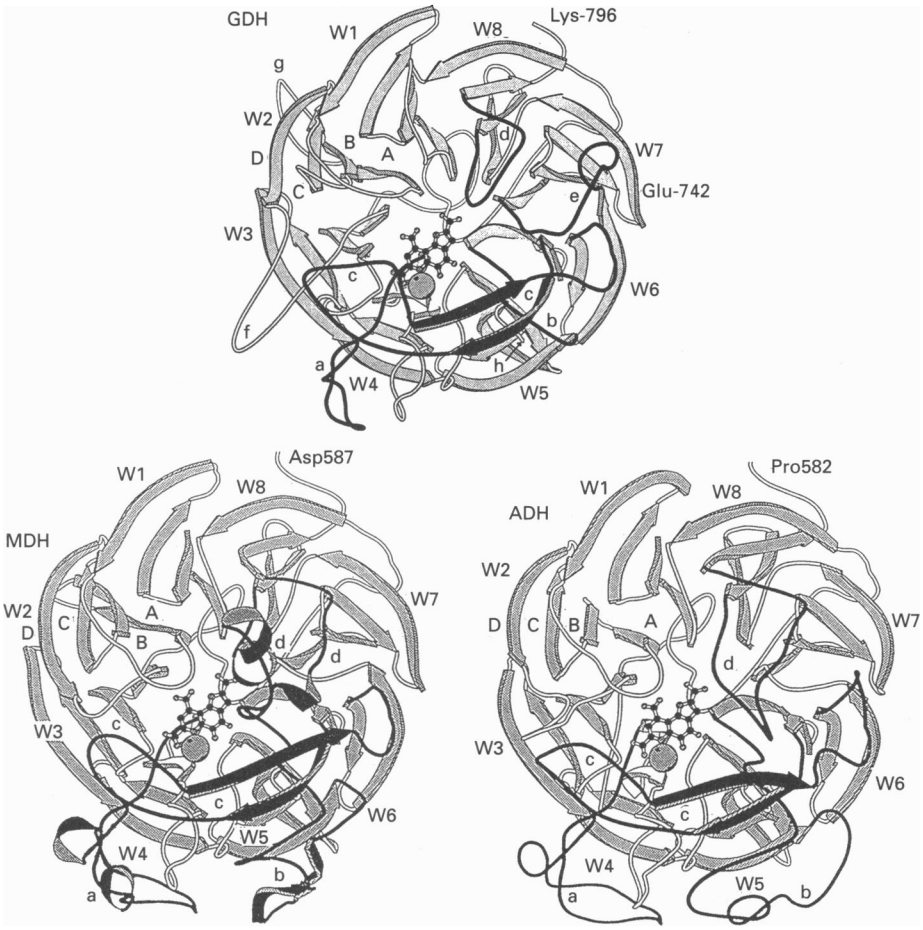

Figure 2 Schematic representation of the backbone of GDH, MDH and ADH showing their major secondary structure

The model GDH structure is of the C-terminal section of the membrane-bound GDH [19] (residues 155–796). The model ADH structure is of the N-terminal region of the quinohaemoprotein subunit I of the membrane complex [37] (residues 1–590). The MDH is the structure previously published for the α-subunit [3,18]. The prosthetic group is shown as a ball and stick structure, and the Ca^{2+} as a van der Waal's sphere. The β-sheet regions (the W motifs or propellor blades) are labelled W1–W8, and the individual β-strands are labelled A–D, with strand A being closest to the symmetry axis. The major loops (in black) are: a, residues 382–404 (GDH), 201–243 (MDH) and 205–245 (ADH); b, 597–599 (GDH), 352–386 (MDH) and 356–389 (ADH); c, 628–668 (GDH), 415–451 (MDH) and 418–460 (ADH); d, 774–783 (GDH), 537–570 (MDH) and 546–565 (ADH); e, 690–711 (GDH), absent from MDH and ADH. Loops f (320–332) and g (281–284) are only present in GDH. h shows the position where residues 497–579 (GDH) would join the main superbarrel structure. These residues are not present in MDH or ADH and the sequence is too long to model. The Figures were generated with Molscript [38].

motifs made up of 11-residue consensus sequences. These form a planar stabilizing girdle of interactions around the periphery of the subunit [18]. Figure 3 shows the 11-residue sequences that form the docking motifs, and the consensus sequences for GDH, MDH and ADH (see refs. [18] and [37] for structures of these docking motifs). The only difference in the consensus sequences of the three proteins is that some carboxylates at position 8 in MDH are replaced by lysine in GDH and ADH.

Before the structure of MDH had been determined it was suggested that a region showing the greatest sequence identity in quinoproteins might represent a PQQ-binding domain (residues 477–539 in MDH, 713–776 in GDH; 41% identity) [3,17,39]. However, the reason for this higher degree of identity is not obvious because this sequence constitutes the whole W7 motif plus the A and B strands of W8, which are very similar in MDH and GDH, but are not involved in the active site. Site-directed mutagenesis of *E. coli* was used to prepare a mutant altered in this region (Glu-742 modified to lysine) [40]; this residue lies on the outside of the superbarrel (Figure 2). Surprisingly, the modified enzyme was about twice as stable to inhibition by EDTA, which usually inhibits GDH by removing the bivalent ion presumed to be required for PQQ binding. It was not, however, as stable as the GDH of *A. calcoaceticus* in which this residue in the wild-type is a lysine. The authors therefore concluded that this residue is not the basis of the much greater stability of the *A. calcoaceticus* enzyme.

Although two of the three *cis* prolines in MDH are conserved in ADH [37], none is conserved in GDH. Pro-72 of MDH is replaced by His-233 in GDH; this is on a turn which is smaller in GDH than in MDH. Pro-264 of MDH is replaced by Ile-427 in GDH; this occurs on a small loop between B4 and C4, which was modified slightly to accommodate the normal *trans* peptide in GDH. The final *cis* proline in MDH (Pro-387) is replaced by Asn-600 in GDH; this is at the end of the external loop b which is much smaller in GDH (Figure 2).

There are six cysteines in the periplasmic region of GDH, giving the possibility of three disulphides bridges, consistent with the lack of demonstrable thiol groups in the GDH of *E. coli* [12].

Position... / Motif	1	2	3	4	5	6	7	8	9	10	11
W1	Ala-238	Leu	Asp	Ala	Ala	Ser	Gly	Lys	Glu	Lys	Trp-248
W2	Ala-301	Ile	Asn	Ala	Glu	Asn	Gly	Lys	Leu	Cys	Glu-311
W3	Glu-367	Phe	Asp	Val	Asn	Thr	Gly	Glu	Leu	Leu	Trp-377
W4	Ala-445	Leu	Asn	Ala	Thr	Thr	Gly	Lys	Leu	Ala	Trp-455
W5	Val-582	Phe	Pro	Gly	Asn	Leu	Gly	Met	Phe	Glu	Trp-592
W6	Ala-674	Leu	Asp	Leu	Lys	Thr	Asn	Glu	Val	Val	Trp-684
W7	Ala-735	Tyr	Asn	Met	Ser	Asn	Gly	Glu	Lys	Leu	Trp-745
W8	Ala-790	Leu	Pro	Asp	Asp-794	Lys-193	Leu	Lys	Glu	Ala	Trp-198
GDH consensus	Ala	X	Asp/Asn	X	X	Thr	Gly	Lys/Glu	X	X	Trp
MDH consensus	Ala	X	Asp/Asn	X	X	Thr	Gly	Asp/Glu	X	X	Trp
ADH consensus	Ala	X	Asp/Asn	Ala	X	Thr	Gly	Lys/Glu	X	Leu/Val	Trp

Figure 3 Sequences of the tryptophan-docking motifs in the model GDH

This docking occurs at the C–D corners at the end of the C strands and the beginning of the D strands of each W motif; there are no loops between these strands. The consensus sequence for the GDH tryptophan-docking motifs is shown, along with those of MDH (from ref. [18]) and ADH (from ref. [37]). The tryptophan in position 11 forms a stacking interaction between alanine (position 1) on the same motif and the peptide bond between positions 6 and 7 on the following motif. This interaction was first described for MDH by F. Scott Mathews at the 3rd Symposium on PQQ and Quinoproteins (1994) and has been described in full in refs. [18] and [37].

Of the three potential disulphide bridges, two could be modelled satisfactorily. The first is Cys-230/Cys-265, joining strand B1 to the small loop between strands D1 and A2, and the second joins strands B2 and D2. The third bridge involves Cys-664 at the end of loop c, which is in a position that would allow the disulphide bond, but its presumed partner (Cys-559) is in the large unmodelled loop between residues 497 and 579. MDH has two disulphide bridges, one at the active site (Cys-103/Cys-104; also present in ADH) and another between Cys-386 and Cys 415. In GDH only one of these four cysteines is conserved (Cys-265 in GDH; Cys-103 in MDH). Cys-103 is one of the adjacent cysteines that forms the novel disulphide ring structure in MDH. The equivalent sequence in GDH is five residues shorter and Cys-265 lies well away from the PQQ and is able to form a disulphide bridge with Cys-230. This result is consistent with an earlier suggestion that the disulphide ring in MDH is specifically involved in electron transfer from PQQ to the haem of cytochrome c_L [3,41], although there is now some evidence against this function in MDH [26].

The regions where GDH and MDH differ most are the loop regions between the B and C strands within the β-sheet W motifs, and between the D and A strands of adjacent motifs (Figure 2). This was also shown for the model ADH [37], and some of these loop regions show more similarities between GDH and ADH than to MDH. These regions are discussed in the context of their functions below.

The loops on the sides of the superbarrel

Loop a (201–243 in MDH and 382–404 in GDH; Figure 2) shows little sequence identity and is 20 residues shorter in GDH. However, the N-terminal residue of this loop is the tryptophan that forms the floor of the active site (Trp-243 in MDH and Trp-404 in GDH). Loop a in ADH shows little sequence similarity to the equivalent loops in GDH and MDH, but is a similar size to that in MDH, and it also terminates in the tryptophan forming the base of the active-site chamber.

In MDH and ADH there is a large loop (loop b; residues 352–386 in MDH) which is represented by a very short sequence (597–599) in GDH.

As seen in Figure 1, GDH has an 83-residue region (497–579) that is absent from MDH and ADH. These 83 residues, joining strands B5 to C5, constitute too long a sequence for its structure to be modelled, but the positions of the two ends of the sequence (labelled h in Figure 2) allow this region to form another external

loop which could occupy the region occupied by loop b of MDH (absent from GDH; see above).

Possible subunit interactions in GDH

In MDH the α-subunits interact with each other over a large planar interface involving hydrophobic and hydrophilic side-chain interactions of the D strands of W7 and W8, hydrophobic stacking interactions (Pro-42/Pro-42 and Leu-51/Leu-51) and the last ten C-terminal residues (590–599) [18]. These ten residues are absent from GDH (Figure 1) and the residues involved in stacking reactions are not conserved. Although there is some sequence similarity between the D7 and D8 strands of GDH and MDH, these results are consistent with the experimental conclusions that the enzyme, after solubilization from the membrane, appears to be monomeric [10,12]. ADH showed no homology with MDH in these regions, so it was concluded that the model structure agreed with the experimental observations that ADH consists of only one subunit of each of its three component proteins.

Neither GDH nor ADH have been reported to have a small β-subunit equivalent to that in MDH (8.5 kDa). In MDH the β-chain runs across the surface of the α-subunit, making contact all along its length with the edges of the W1–W4 motifs by way of ion-pair interactions involving Glu-148, Glu-193, Arg-197, Lys-236, Glu-267 and Glu-301 [18]. In the GDH model only Glu-373 (Glu-193 of MDH) could be involved in ionic interactions, consistent with the observed lack of an equivalent β-subunit in GDH. No convincing specific role for the unusual non-globular β-subunit in MDH has been proposed except that it might act to stabilize the folded form of the large α-chain [3,18]. If this is its function, then perhaps a similar stabilizing role in GDH is provided by its interaction with the transmembrane region of the monomer, and ADH may be stabilized by interaction with the other two proteins in the three-protein complex.

The active-site funnel in the surface

A remarkable feature of the surface of MDH is the shallow hydrophobic funnel that leads to the active site [3]. It involves three separate sequences and consists of the following residues which are poorly conserved in GDH (surface-accessible residues in MDH are given in bold type):

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MDH: 100-AVACCDL;   420-PFMLP;   430-FFV;
GDH: 262-HVTC---;   633-KGTGT;   645-YGV;

MDH: 540-WPGVGLVFDLADPTAGL
GDH: 777-SF----------GTK
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In MDH and ADH the sequence between D1 and A2 (residues 93–109) includes the adjacent cysteines (103–104) which make up the novel disulphide ring at the base of the funnel leading into the active site. In GDH the equivalent region (253–266) forms part of the funnel, but it is smaller and lacks the disulphide ring. Loop c shows a little sequence identity and includes four extra residues, but the hairpin β-structure is retained in GDH and so forms part of the funnel in both proteins, as it also does in ADH (Figure 2; residues 626–668 in GDH; 413–451 in MDH). Loop d (residues 774–783 in GDH; 537–570 in MDH) has negligible sequence similarity and is smaller in GDH and ADH, which both lack the helical structure seen in MDH (Figure 2), and thus have slightly larger funnels at the entrance to the active site. Figure 4 shows the active-site funnel of the model GDH, illustrating the wider entrance into the active site in this enzyme compared with that in

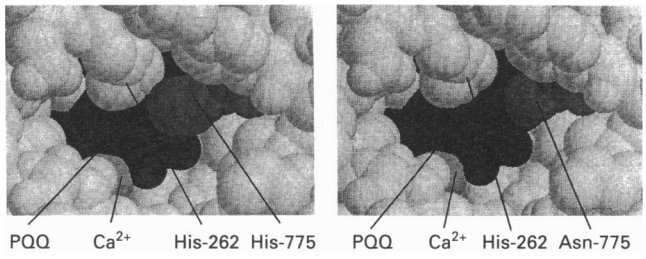

Figure 4 Spacefill view of the active-site funnel of the model GDH, and GDH in which His-775 has been replaced by asparagine

Replacement of this histidine by asparagine in the GDH of *G. oxydans* leads to an enzyme able to oxidize maltose, thus producing a bacterial strain that has acquired the ability to grow on this disaccharide. This Figure shows the comparison of the active-site funnel of both the original model GDH and the Quanta-generated modification containing asparagine in its place. The position of His-262, which may play a similar role to the novel disulphide ring structure of MDH in maintaining the position of the PQQ [26], is also indicated.

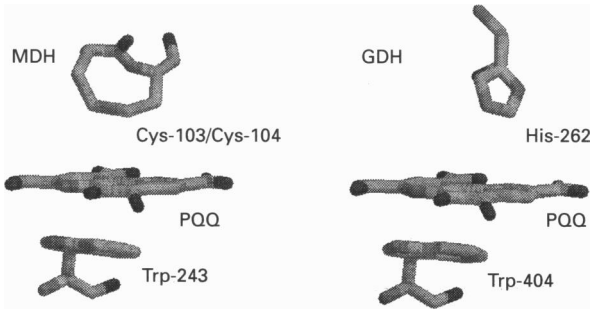

Figure 5 Comparison of the stacking interactions of the PQQ in MDH and the model GDH

In MDH the PQQ is stacked between the co-planar Trp-243 and the novel disulphide ring system of Cys-103 and Cys-104 [18]. In GDH the co-planar tryptophan is retained (Trp-404) but the disulphide is not conserved. Instead, His-262 may perform a similar role in helping to bind the PQQ into the active-site region. The C-4 carbonyl oxygen is shown out of the plane of the ring as in MDH, but there is no evidence that this semiquinone structure is ever present in GDH.

MDH [3]. His-775 (which is conserved in all GDHs) partly obscures access to the active site and it is probably the presence of this residue that determines the specificity for monosaccharides; in *G. oxydans*, GDH normally only oxidizes monosaccharides but a naturally occurring strain has been isolated that can oxidize the disaccharide maltose [31]. In the GDH isolated from this strain there has been a single-base-pair substitution, leading to the replacement of this histidine by asparagine which clearly leads to a more accessible active site (Figure 4).

There is an extra loop in GDH (loop e in Figure 2; residues 691–711) which may also form an important part of the funnel in GDH. However, as this loop is 21 residues long, the modelling cannot be accurate and, although it fits into the space available in the model GDH, it is not possible to predict its importance in the funnel region.

This surface funnel region shows the greatest difference between the two proteins, mainly because of the absence of the hydrophobic helical structure in GDH that is so prominent in MDH. This is the part of the surface nearest to the prosthetic group and is an obvious region for interaction of MDH with its electron acceptor cytochrome c_L.

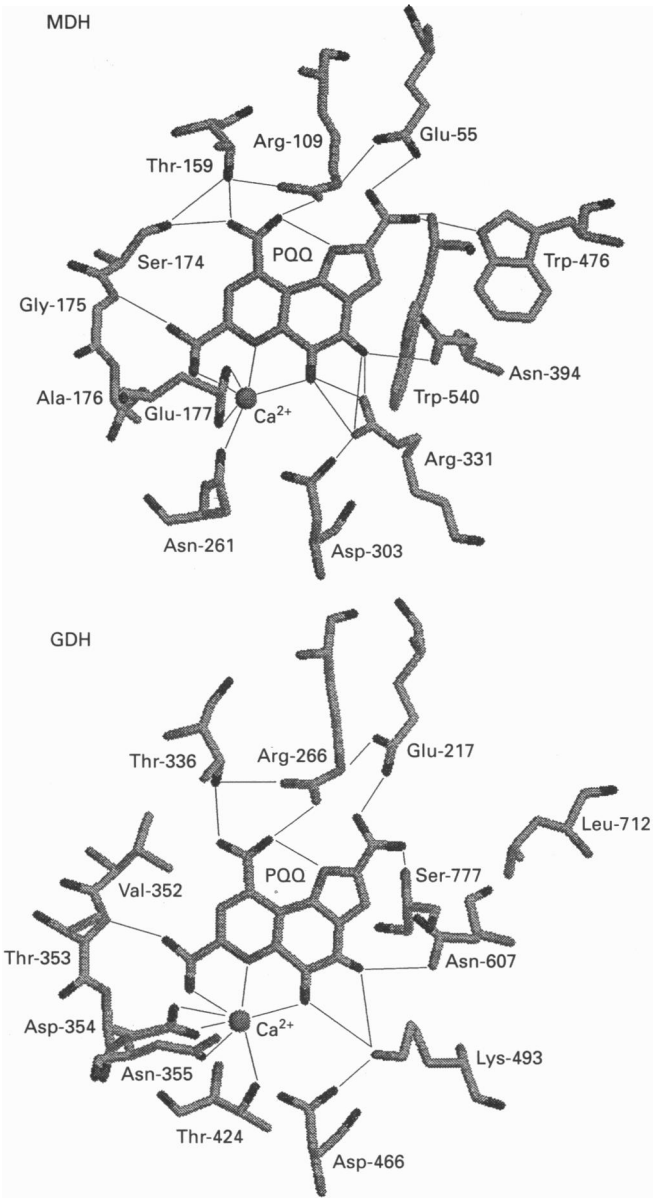

Figure 6 Comparison of the co-ordination of Ca^{2+} and the bonding of PQQ in the active site of MDH and the model GDH

Of the equatorial interactions with PQQ the significant differences between the two enzymes are that residues Ser-174, Arg-331 and Trp-476 of MDH are replaced by Val-352, Lys-493 and Leu-712 in GDH; this results in fewer hydrogen bonds to the PQQ in GDH. Ca^{2+} is included in the model GDH, although this may be replaced by Mg^{2+} in the GDH from some bacteria. By analogy with the mechanism proposed for MDH [3], Asp-466 may act as a base, initiating the reaction by abstraction of a proton from glucose; in this mechanism the Ca^{2+} acts as a Lewis acid, co-ordinating with the C-5 carbonyl oxygen, which gives rise to the electrophilic C-5 carbon of PQQ. Asn-402 in GDH (Thr-241 in MDH) has been omitted to achieve greater clarity.

The active-site region in the model GDH

In MDH [3,18] and the model ADH [37], the PQQ ring is held in position between the indole ring of a tryptophan (Trp-243 in MDH, Trp-245 in ADH) and the two sulphur atoms of the disulphide ring structure. The model structure of GDH retains the tryptophan (Trp-404), but lacks the disulphide ring which is replaced by His-262 (conserved in all GDHs) (Figure 5). This suggests that the function of His-262 may be to maintain the position of PQQ in the active site, a suggestion that is consistent with the demonstration by chemical modification that a histidine residue is essential for binding PQQ in the GDH of *Ps. fluorescens* [25].

In addition to the stacking reactions with PQQ there are many equatorial interactions between substituent groups of the PQQ ring system and amino acid residues, mainly on the A strands of the β-sheets of the superbarrel in MDH; many of these interactions are conserved in GDH (Figure 6). The residues in GDH involved in the equatorial reactions are Glu-217, Arg-266, Thr-336, Val-352 (Ser-174 in MDH), Thr-353 (Gly-175 in MDH), Asp-354 (Ala-176 in MDH), Asn-402 (Thr-241 in MDH), Lys-493 (Arg-331 in MDH), Asn-607, Leu-712 (Trp-476 in MDH) and Ser-777 (Trp-540 in MDH). The proposal that Arg-266 is involved in binding the 9-carboxylate of PQQ is consistent with the previous conclusion of Imanaga [25] based on inhibition by 8-anilino-1-naphthalenesulphonate of binding of PQQ to apoenzyme. It is also consistent with the previous demonstration that the 9-carboxylate is essential for binding PQQ into the active site of the GDH from *E. coli* [42]. As seen in Figure 6, there are relatively fewer equatorial PQQ interactions in GDH which, together with the replacement of the disulphide ring by histidine, may explain why it is possible to release PQQ from GDH but not from MDH, or from ADH in which the interactions with PQQ are similar to those in MDH [37].

In MDH the co-ordination sphere of the Ca^{2+} in the active site contains both PQQ and protein atoms. From the protein, both oxygens of the carboxylate of Glu-177 and the amide oxygen of Asn-261 are involved, and the PQQ donor atoms include the C-5 carbonyl oxygen, one oxygen of the C-7 carboxylate and, remarkably, the N-6 ring atom. In the active site of GDH, the PQQ ligation to Ca^{2+} is likely to be the same as in MDH, as previously proposed by Imanaga [25]. The ligation to the protein must be different from that in MDH: Glu-177 and Asn-261 in MDH are replaced by Asn-355 and Thr-424 in the model GDH which can only provide two interactions; however, Asp-354 is close enough to form another two bonds (Figure 6). These three residues are conserved in the GDHs the sequences of which are known (Figure 1), and an alternative suggested ligation of cysteine and tyrosine to the Ca^{2+} in GDH of *Ps. fluorescens* [25] is unlikely. Beside its role in maintaining the position of PQQ in the active site, it has been suggested that in MDH the Ca^{2+} ion might act as a Lewis acid, through its co-ordination with the C-5 carbonyl oxygen, thus providing the electrophilic C-5 for attack by an oxyanion or hydride from the substrate [2,3]. In this way the Ca^{2+} could play a dual role in structure and catalysis. The observation that Mg^{2+} can replace Ca^{2+} for reconstitution of active enzyme from apoenzyme plus PQQ in some GDHs raises the possibility that in these GDHs the co-ordination of the cation at the active site might be different.

Figure 6 shows that Asp-466 (which is conserved in all GDHs) occupies the same position in the model GDH as Asp-303 occupies in MDH. This is of importance because it has been suggested that this residue has the role of an active-site base, initiating reaction with the alcohol substrate by proton abstraction [2,3,18]. This aspartate is conserved in MDH, ADH and the three GDHs, therefore supporting the observation that it is important in the mechanism. Two types of mechanism have been discussed for MDH, both involving an initial proton abstraction. This is followed by attack on the electrophilic C-5 of PQQ by the oxyanion to form a hemiketal intermediate [3,43]; or attack by a hydride from the alcohol oxyanion, leading directly to formation of the aldehyde and the quinol form of PQQ [3]. Either of these mechanisms could operate with glucose, the product being the gluconolactone. It should be noted that a

previous suggestion [25], that the reaction with glucose is initiated by proton abstraction by a cysteine residue, is unlikely in the GDHs whose sequences are known because there is no cysteine within the appropriate region of the active site, and all cysteines are involved in disulphide bridges.

The interaction of GDH with ubiquinone

In MDH it has been suggested that the natural electron acceptor (cytochrome c_L) may bind in the hydrophobic funnel region of MDH, thus offering the shortest path for electron transfer from the quinol prosthetic group ($PQQH_2$) to the haem of cytochrome c_L [3,44,45], by way of two separate electron-transfer steps and a semiquinone intermediate form of PQQ [3]. By contrast, the natural electron acceptor for GDH is the hydrogen carrier ubiquinone in the periplasmic membrane, and electrons must pass from $PQQH_2$ to the ubiquinone to give the ubiquinol. In GDH the active-site funnel is not hydrophobic and there is no suggestion from the model structure or from the primary sequence that there is a hydrophobic region of the protein that could interact with the membrane, except for the N-terminal transmembrane segments. It has been proposed that the binding site for ubiquinone is in a loop in the periplasmic face of the N-terminal transmembrane region (residues 1–154) [19,46]. It is possible that this region interfaces with the active-site funnel in GDH, but the possibility that the electron-transfer route involves the large loops on the surface of GDH which could not be modelled in this investigation cannot be ruled out.

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